

Molecular cloning, sequence analysis, and cadmium stress-rated expression changes of BTG1 in freshwater pearl mussel (*Hyriopsis schlegelii*)

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Abstract: The B cells translocation gene 1 (*BTG1*) is a member of the BTG/TOB family of anti-proliferative genes, which have recently emerged as important regulators of cell growth and differentiation among vertebrates. Here, for the first time we cloned the full-length cDNA sequence of *Hyriopsis schlegelii* (*Hs-BTG1*), an economically important freshwater shellfish and potential indicator of environmental heavy metal pollution, for the first time. Using rapid amplification of cDNA ends (RACE) together with splicing the EST sequence from a haemocyte cDNA library, we found that *Hs-BTG1* contains a 525 bp open reading frame (ORF) encoding a 174 amino-acid polypeptide, a 306 bp 5' untranslated region (5' UTR), and a 571 bp 3' UTR with a Poly(A) tail as well as a transcription termination signal (AATAAA). Homologue searching against GenBank revealed that *Hs-BTG1* was closest to *Crassostrea gigas* *BTG1*, sharing 50.57% of protein identities. *Hs-BTG1* also shares some typical features of the BTG/TOB family, possessing two well-conserved A and B boxes. Clustering analysis of *Hs-BTG1* and other known BTGs showed that *Hs-BTG1* was also closely related to *BTG1* of *C. gigas* from the invertebrate BTG1 clade. Function prediction via homology modeling showed that both *Hs-BTG1* and *C. gigas* *BTG1* share a similar three-dimensional structure with *Homo sapiens* *BTG1*. Tissue-specific expression analysis of the *Hs-BTG1* via real-time PCR showed that the transcripts were constitutively expressed, with the highest levels in the hepatopancreas and gills, and the lowest in both haemocyte and muscle tissue. Expression levels of *Hs-BTG1* in hepatopancreas (2.03-fold), mantle (2.07-fold), kidney (2.2-fold) and haemocyte (2.5-fold) were enhanced by cadmium (Cd^{2+}) stress, suggesting that *Hs-BTG1* may have played a significant role in *H. schlegelii* adaptation to adverse environmental conditions.

Keywords: *Hyriopsis schlegelii*; *BTG1*; Gene cloning; mRNA expression; Cadmium stress

Exposure to heavy metals—cadmium in particular—is widely known to be toxic to humans and higher vertebrates, negatively affecting several key organs. Unfortunately, industrial pollution run-off often results in these metals making their way into water sources, where they are absorbed by the ecosystem's resident species. For example, oysters were previously found to accumulate environmental cadmium, suggesting that they may serve as potential indicator organisms for marine cadmium contamination (Lu et al, 1998). However, the toxicological activities and effects of such heavy metals among invertebrates such as freshwater shellfish are not well understood. This situation is especially acute in developing countries and regions such as China, where heavy levels of water pollution that has

accompanied industrial development.

One key reason that shellfish species like oysters can serve as viable indicators of environmental heavy metals is that they have remarkable adaptability to

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different environments and are resistant to the toxic effects of metals like cadmium. Among freshwater shellfish, both the Japanese *Hyriopsis schlegelii* (*H. schlegelii*) and Chinese *H. cumingii* species of mussels exhibit similar characteristics. In 1997, *H. schlegelii* was introduced to China by the Reservoir Development Company in Fuzhou City, Jiangxi Province. Over the last decade, this species has become a key economic feature of freshwater shellfish production, largely due to decades of selective breeding that have results in strong disease resistance and environmental adaptability, as well as its enlarged shell width and other physical characteristics contributing to senior pearls cultivation (He et al, 2013; Peng et al, 2012a, b; Xie et al, 2011). In order to develop a better ecological farming, exploring molecular indicator capable of monitoring environmental pollution is particularly important for this mussel.

Testing for the presence and effects of cadmium among potential indicator species is not always straightforward, but gene expression analysis under various environmental factors stress is a commonly employed experimental method to explore candidate gene's corresponding function (Duan et al, 2013; Wang et al, 2011) and in doing so illustrating the effects of certain environmental pollutants on different organisms. In the present study, we sought to examine the *H. schlegelii* *B cell translocation gene 1* (*BTG1*), which has been cloned and studied in several species, including *Rattus norvegicus*, *Mus musculus*, *Gallus gallus*, *Xenopus laevis*, and *Danio rerio* (Fu et al, 2012), but not among many lower animals. *BTG1* is a member of the BTG/TOB family that was identified from B lymphoblastic leukemia with chromosomal translocation (Rouault et al, 1992). The BTG/TOB family is a class of anti-proliferative proteins involved in negative regulation of cell cycle (Winkler, 2010) and tumor cell growth (Mauxion et al, 2009). BTG/TOB proteins have a conservative BTG region of 100 – 120 amino acids at its amino-terminal (N terminal), containing two well-conserved A and B boxes (Mauxion et al, 2009; Winkler, 2010). Among mammals, the BTG/TOB family includes six members: BTG1, BTG2/PC3/Tis21, BTG3/ANA, BTG4/PC3B, TOB1/TOB, and TOB2 (Winkler, 2010), with both BTG1 and BTG2 belonging to the same subfamily with an approximate carboxyl terminal (C terminal) length (Mauxion et al, 2009). Previously, the BTG/TOB proteins were reported to be involved in several different physiological activities,

including regulation of embryonic development, cell differentiation and apoptosis, among others (Mauxion et al, 2009). Similarly, BTG/TOB proteins were also found to affect gene transcription and mRNA stability in cells via deadenylation (Yang et al, 2008; Mauxion et al, 2009). In this study, we obtained the full length cDNA of *Hs-BTG1* by library screening and RACE cloning to investigate whether BTG1 is involved in the response against heavy metal stress.

MATERIALS AND METHODS

Experimental materials and reagents

We obtained healthy *H. schlegelii* from the national seed market of *Hyriopsis* at Reservoir Development Company in Fuzhou City, Jiangxi Province. The *H. schlegelii*, with a shell length of 107±6.5 mm, was cultured in a laboratory aquarium for one week (18-25 °C) prior to testing.

RNAase inhibitors, DNA Polymerases and Markers were purchased from TaKaRa. Gel extraction kit and plasmid extraction kit were purchased from Axygen, while DNase I (RNase Free) and a first strand cDNA synthesis kit were purchased from Promega. TRIzol was from Invitrogen. *E. coli* DH5α cells were stored in our lab. Primers were synthesized by Shanghai Sangon Company. Cadmium chloride (CdCl₂·5H₂O, AR) was produced by the Shanghai Chemical Reagent and other conventional reagents of analytical grade were obtained from Sinopharm Group.

cDNA

Total RNA of different tissues was extracted using TRIzol prior to assessing its purity and concentration. Primary sample digestion by RNase-free DNase I was performed before cDNA synthesis. The organization-specific cDNA synthesis was conducted as described previously (He et al, 2013; Peng et al, 2012a, b).

cDNA library screening

We performed PCR amplification of monoclonal colony using random primers (M13) according to our established cDNA library from blood cells of *H. schlegelii* (Xie et al, 2011), under the following conditions: 94 °C 5 min; 94 °C 30 s, 45 °C 30 s, 72 °C 90 s, 33 cycles; 72 °C 7 min. Amplified fragments more than 500 bp in length were sequenced using M13 sequencing primers (Sangon), and then we obtained expressed sequence tags (ESTs) sequence of *Hs-BTG1*.

Full-Length cDNA cloning and molecular characterization

The 3' and 5' cDNA ends were amplified using SMART™ RACE Amplification Kit and Advantage 2 PCR Kit. Primers (*BTG1*-GSP1 and *BTG1*-GSP2; shown in Table 1) were designed using Primer Premier 5.0 according to the ESTs sequence of *Hs-BTG1*. Touchdown PCR amplification was performed using universal primers (UPM), 5' specific (*BTG1*-GSP1) and 3' specific (*BTG1*-GSP2) primers, with 5'-RACE-Ready and 3'-RACE-Ready cDNA as the template. PCR reaction system was as follows: 10× Advantage 2 PCR Buffer 5 µL, dNTP Mix 1 µL, 5'-RACE-Ready cDNA or 3'-RACE-Ready cDNA 2.5 µL, UPM 5 µL, *BTG1*-GSP1 or *BTG1*-GSP2 1 µL, Advantage 2 Polymerase Mix 1 µL, PCR-Grade water 34.5 µL, total volume 50 µL. Reaction

conditions were as follows: 94 °C 5 min; 94 °C 30 s, 72 °C 3 min, 5 cycles; 94 °C 30 s, 70 °C 30 s, 72 °C 3 min, 5 cycles; 94 °C 30 s, 68 °C 30 s, 72 °C 3 min, 28 cycles; 72 °C 10 min. Amplified products were recovered and purified using a gel extraction kit before being sequenced by Shanghai Sangon Company.

NCBI ORF Finder and BlastP were used for identifying the largest ORF and homologous amino acid sequences of *Hs-BTG*. SignalP 4.0 was used for predicting the signal peptide. ClustalW was used in multiple comparisons of the amino acid sequences from different species. Protein homology modeling was conducted by using SWISS-MODEL. Chimera 1.8 was used for the tertiary structure analysis. The phylogenetic tree was constructed using Neighbor-Joining (NJ) method with 1000 bootstraps in Mega 4.1.

Table 1 Primers used for cloning *Hyriopsis schlegelii* *BTG1* cDNA and expression quantification

Primer name	Sequence (5'–3')
M13 F	TGTAACGACGCGCCAGT
M13 R	CAGCAACAGCTATGAC
SMART II™ A Oligonucleotide (12 µmol/L)	AAGCAGTGGTATCAACGCAGAGTACGCGGG
3' -RACE CDS Primer A (3'-CDS; 12 µmol/L)	(T) ₂₅ V N (N=A, C, G, or T; V=A, G, or C)
5' -RACE CDS Primer A (5' -CDS; 12 µmol/L)	AAGCAGTGGTATCAACGCAGAGTAC(T) ₃₀ V N
10×Universal Primer A Mix (UPM)	(N=A, C, G, or T; V=A, G, or C)
Long Primer (0.4 µmol/L)	CTAATACGACTCACTATAGGGCAAGCAGTGGTA
Short Primer(2 µmol/L)	TCAACGCAGAGT
<i>Hs-BTG1</i> 5GSP1(10 µmol/L)	CTAATACGACTCACTATAGGGC
<i>Hs-BTG1</i> 3GSP2(10 µmol/L)	CGTGGTCCTCGTGCGAAATAGAATG
<i>Hs-BTG1</i> qPCR-F	TGCCACTTTGGTTTCCAGTTGAGG
<i>Hs-BTG1</i> qPCR-R	GCTGGCTTATCGTGTGGAAT
β -actin qPCR-F	CGGTACTGGCATCCTCTGAT
β -actin qPCR-R	AAGGTTACGCCCTTCCTCAT
	GCCATTTCCTGCTCAAAGTC

Expression profile of *Hs-BTG1*

Totally, we tested 10 tissues from three mussels, including intestines, gills, gonads, mantle, ax foot, adductor muscle, blood cells, hepatopancreas, kidneys, and hearts. Total RNA was extracted and treated by RNase-free DNase I (Promega). 1.5 µg total RNAs were reverse transcribed using MMLV RT Kit (Promega) under following reaction conditions: 37 °C, 60 min, 75 °C 5 min, 4 °C, hold. Expression profiles of *BTG1* from different tissues were detected by quantitative PCR using SYBR Premix Ex Taq II kit (TaKaRa) with β -actin as an

internal reference. The amplification condition was as follows: 95 °C 5 min; 95 °C 30 s, 60 °C 30 s, 72 °C 30 s, 40 cycles; 72 °C 10 min. $2^{-\Delta\Delta CT}$ relative quantification method was used. Tissues that showed the lowest expression were set as controls. Each sample had three replicates, and all experiments were repeated in triplicate.

Cadmium (Cd²⁺) stress test

We determined the test concentrations following the method reported by literature (Kim et al, 2012). The standard Cd²⁺ concentration (≤ 0.005 mg/L) according to

"Water quality standard for fishery" GB7471 (0.005 mg/L) as well as 1 000 times the standard concentration (5mg/L) were respectively set as the control and experimental groups. The exposure method was static contact with $\text{CdCl}_2 \cdot 5\text{H}_2\text{O}$ infected water. Totally, 20 healthy mussels in each group were cultured in an aquarium with 20 L test solution. The water was changed by 50% every day and the concentration was maintained during the trial period. We randomly selected 3–4 mussels at exposure points of 0 h, 6 h, 12 h, 24 h, 48 h, 96 h, and from these total RNA from the hepatopancreas, kidneys, blood cells, and mantle was extracted. This experiment was repeated three times.

Target gene expression analysis under Cd^{2+} stress

We performed SYBR green-based real-time quantitative PCR assay (He et al, 2013; Peng et al, 2012a, b) to detect the expression changes of target gene in the 5 mg/L concentration of cadmium stress conditions. The final relative expression result is the product of two relative expression values: the $2^{-\Delta\Delta\text{CT}}$ value of the experimental group (5mg/L) at different time points relative to 0h, and the $2^{-\Delta\Delta\text{CT}}$ value of the experimental group relative to the control group at the same time point. The mRNA expression difference of *Hs-BTG1* was analyzed using SPSS 16.0 (SPSS, inc. Chicago, USA). $P < 0.05$ and $P < 0.01$ were set as significant and highly significant, respectively.

RESULTS

Sequence analysis of *Hs-BTG1*

The full length cDNA of *Hs-BTG1* was found to be 1 402 bp and involves a complete coding sequence of 525 bp, including an ORF encoding 174 amino acids, 306 bp 5'-UTR, 571 bp 3'-UTR, with a typical AATAAA and poly A tail (Figure 1; sequence submitted to NCBI, accession number KF015272). BlastP analysis (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) showed that the Hs-BTG1 has one typical conserved N terminal domain shared by BTG Superfamily (*i.e.*, BTG/TOB domain) which is characterized by two well-conserved boxes named A and B (Figure 1, Figure 2) (Fu et al, 2012; Sakaguchi et al, 2001; Winkler, 2010); SignalP-4.1 prediction identified no signal peptide sequence.

Homology and cluster analysis

Amino acid sequences of BTG1 from *H. schlegelii*, *C. gigas*, *D. rerio*, *X. laevis*, *G. gallus*, *M. musculus*, and

H. sapiens were compared using Clustal W (Figure 2), showing that Hs-BTG1 has the highest identity (50.57%) with BTG1 from *C. gigas*. Hs-BTG1 has lower identity with BTG1 in vertebrates, with an identity of 35.67% with human BTG1. We next performed a homology modeling of BTG1 from *H. schlegelii* and *C. gigas* according to reported 3D structure of human BTG1 protein (Yang et al, 2008; Winkler, 2010), and found each presents a similar 3D structure (Figure 2) with five α -helices and two antiparallel β -sheets.

Cluster analysis (Figure 3) using Mega4.1 (Neighbor-Joining, NJ) showed that BTGs of vertebrates and invertebrates clustered into two branches. In invertebrates, the Hs-BTG1 was clustered firstly together with the *C. gigas* BTG1, and then BTG1 of *L. gigantea*, *L. Singoriensis*, *A. florae*, and *C. formosanus* along with the aforementioned two BTG1 were clustered into one large branch. In vertebrates, another large branch was also formed mainly by some BTG1s from fish to humans, as well as BTG2 from *G. gallus*, *M. musculus*, and *H. sapiens*.

Expression profile of Hs-BTG1

Expression profile of *Hs-BTG1* in different tissues was analyzed via real-time quantitative PCR (RT-qPCR) (Figure 4). Expression of *Hs-BTG1* mRNA was detected in all 10 tissues—intestines, gills, gonads, mantle, ax foot, adductor muscle, blood cells, hepatopancreas, kidney, and heart—but was most highly expressed in the hepatopancreas and then the gills, with while blood cells exhibited the lowest expression of *Hs-BTG1*.

Expression change under Cadmium stress

Expression changes of *Hs-BTG1* in hepatopancreas, kidneys, blood cells, and mantle under Cadmium (Cd^{2+}) exposure for 0 h, 6 h, 12 h, 24 h, 48 h, 96 h were shown in Figure 5. In the hepatopancreas, *Hs-BTG1* expression significantly increased at 6 h, decreased at 12 h, then increased at 24 h, reaching a peak at 48 h (2.03-fold of the standard control group) and then falling back at 96h. The overall expression pattern in blood cells was quite similar, with the only difference being a continued decrease at 24 h, though the expression level also peaked at 48h (2.5-fold of the standard control group). In kidney tissue, the *Hs-BTG1* transcription dropped until an increase at 24 h (2.2-fold of the standard control group) before slowly returning to normal levels between 48 h-96 h. Similarly, *Hs-BTG1* expression level in mantle also reached the peak at 24 h (2.07-fold of the standard control

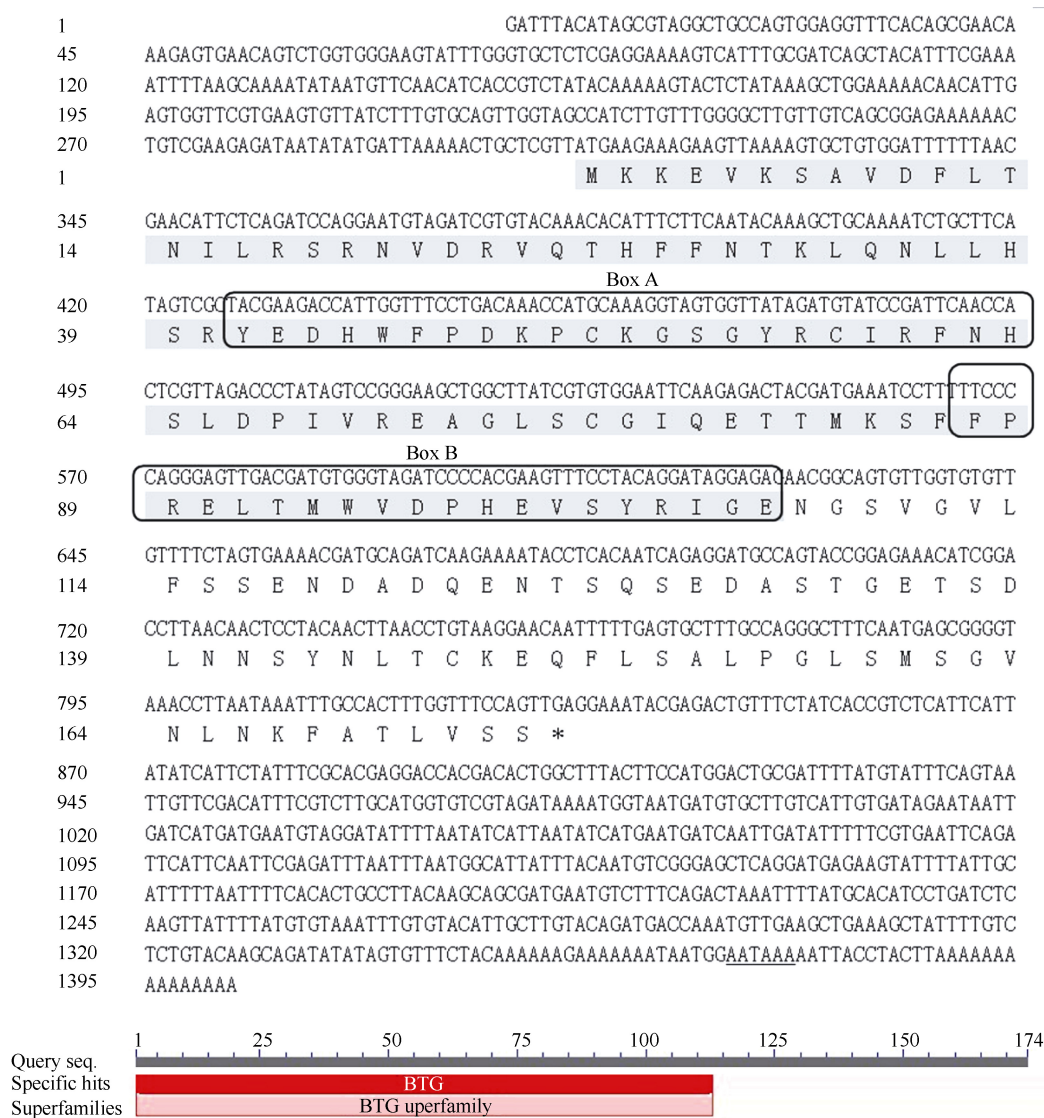


Figure 1 cDNA and deduced amino acid sequence of BTG1 in *Hyriopsis schlegelii*

Nucleotide and deduced amino acid sequence were numbered on the left. The BTG/TOB function domain was shaded and the two highly conserved domains (BTG Boxes A and B) were indicated with a rounded rectangle. The stop codon TGA and the polyadenylation signal AATAAA were tagged with an asterisk and underline, respectively. The lower image depicts the BlastP result for Hs-BTG1.

group), but decreased at 48 h, and then showed a slight increase at 96 h.

DISCUSSION

To date, there are 6 identified members of the BTG/TOB family in vertebrates, named for its BTG tag sequence in the N terminal. The BTG region contains two highly conserved homologous short motifs, namely A box (YKHHWFPDRPNKSGYRCIRIN) and B box (LPSELTLWVDPFEVSYRIGE). These two boxes are separated by 20–25 nonconservative amino acids (Guehenneux et al, 1997; Sakaguchi et al, 2001; Fu et al,

2012). The A box seems to have an anti-proliferation function, while the B box appears act as the binding sites of its target molecule (Yang et al, 2008). Among mammals, the BTG/TOB family proteins can be classified into 3 subfamilies based on different sequence length of the C terminal: BTG1 and BTG2 as a subgroup, BTG3 and BTG4 as another subfamily, while TOB1 and TOB2 as the third subgroup (Matsuda et al, 2001; Mauxion et al, 2009; Winkler, 2010), with both BTG1 and BTG2 having a shorter C terminal and an extra 8–10 amino acids in the N terminal compared with other two subgroups (Winkler, 2010).

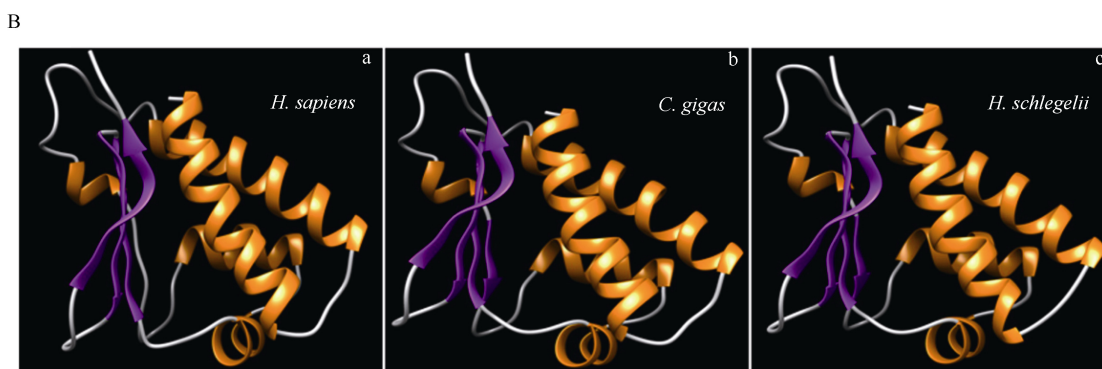
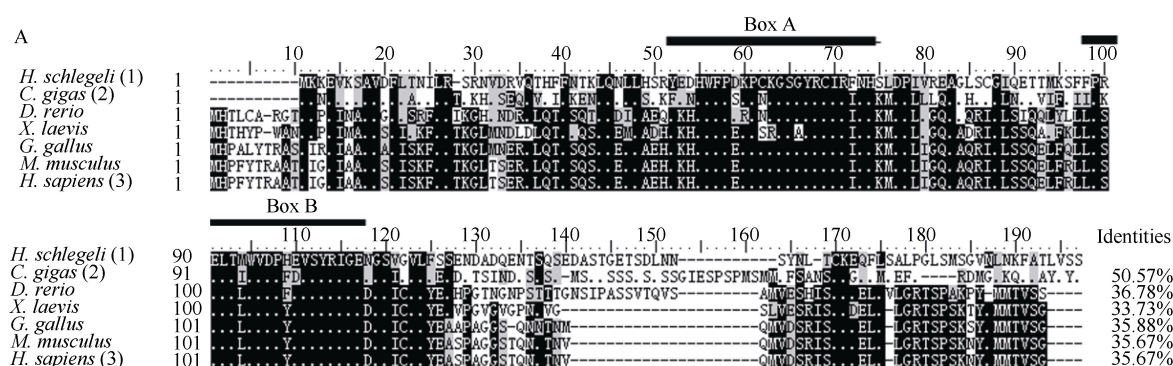


Figure 2 Multiple alignment of BTG1 between *Hyriopsis schlegelii* with other species

(A) Alignment of the deduced amino acid sequence of *H. schlegelii* BTG1 with the corresponding sequences from other species; (B) the tertiary (3D) structures of *Homo sapiens* BTG1 (a), *Crassostrea gigas* BTG1 (b), and *H. schlegelii* BTG1 (c) modeled in SWISS-MODEL. The highly conserved A Box and B Box regions are marked in (A) and a schematic representation of the α -helical (yellow) and β -sheet (purple) structural elements are present in (B). a. *H. sapiens*: NP_001722.1 BTG1, b. *C. gigas*: EKC27510.1 BTG1, c. *H. schlegelii* BTG1: AGT79958.1; *C. gigas*: EKC27510.1; *Danio rerio*: NP_956314.1; *Xenopus laevis*: NP_001080825; *Gallus gallus*: CAA45507.1; *Mus musculus*: NP_031595.1; *H. sapiens*: NP_001722.1. “...” indicates the same amino acid; “---” indicates the default at the corresponding sites.

Though *BTG1* is comparatively well characterized in mammals and other vertebrates, little has been done on freshwater shellfish. Here, for the first time, we identified a homologous gene (*Hs-BTG1*) of the BTG/TOB family in *H. schlegelii*. The motif structure of the deduced amino acid sequences also contains highly conserved A and B box. BlastP analysis showed that Hs-BTG1 has the highest identity (50.57%) with *C. gigas* BTG1. Homology modeling further showed that both *H. schlegelii* and *C. gigas* BTG1 present similar 3D structure with the reported human BTG1 protein (Yang et al, 2008; Winkler, 2010). Interestingly, both *H. schlegelii* and *C. gigas* BTG1 have a truncated (for 8–10 amino acids) N terminal as compared with vertebrate BTG1, but the length of their C terminal is roughly consistent with the corresponding part of vertebrate BTG1. We also found that Hs-BTG1 and *C. gigas* BTG1 were clustered together, both belonging to the invertebrate BTG1 large branch, while vertebrate BTG1 and BTG2 fell into another large branch. These

results concur with both BlastP and traditional classification, so consequently the sequence we obtained was classified into the first subgroup in the BTG/TOB family and termed as the homologue (Hs-BTG1) to other species BTG1.

It is worth noting that there are a very limited number of BTG/TOB members identified in invertebrates, making it difficult to accurately position the relationship between Hs-BTG1 and other BTG/TOB members from the constructed cluster tree (Fu et al, 2012), which is supported by the low Bootstrap value in some branches. Additionally, while BTG1 from invertebrates (such as *C. gigas* and *H. schlegelii*) showed relatively low identity compared with vertebrate BTG1, they have conserved motif and similar 3D structure shared by the entire BTG family, implying that BTG1 of lower vertebrate animals may retain some conservative function and also possess different functions as compared to their vertebrate homologs (Matsuda et al, 2001).

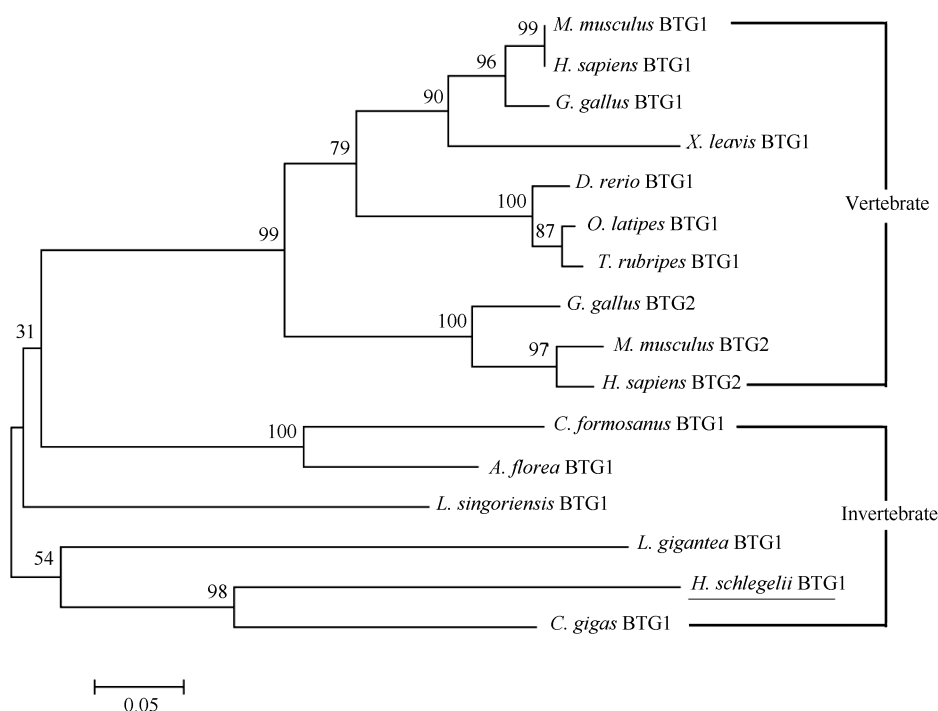


Figure 3 Phylogenetic tree of BTG1 from *Hyriopsis schlegelii* and other animals

Mus musculus BTG1: NP_031595.1; *Homo sapiens* BTG1: NP_001722.1; *Gallus gallus* BTG1: NP_990681.1; *Danio rerio* BTG1: NP_956314.1; *Oryzias latipes* BTG1: XP_004083105.1; *Takifugu rubripes* BTG1: XP_003972839; *Xenopus laevis* BTG1: NP_001080825; *G. gallus* BTG2: XP_418053; *M. musculus* BTG2: NP_031595.1; *H. sapiens* BTG2: NP_006754.1; *Coptotermes formosanus* BTG1: AGM32529; *Apis florea* BTG1: XP_003697190; *Lycosa singoriensis* BTG1: ABX75488; *Lottia gigantea* BTG1: ESO88805; *H. schlegelii* BTG1: AGT79958.1; *C. gigas* BTG1: EKC27510.1. The number in the branch is the bootstrap value.

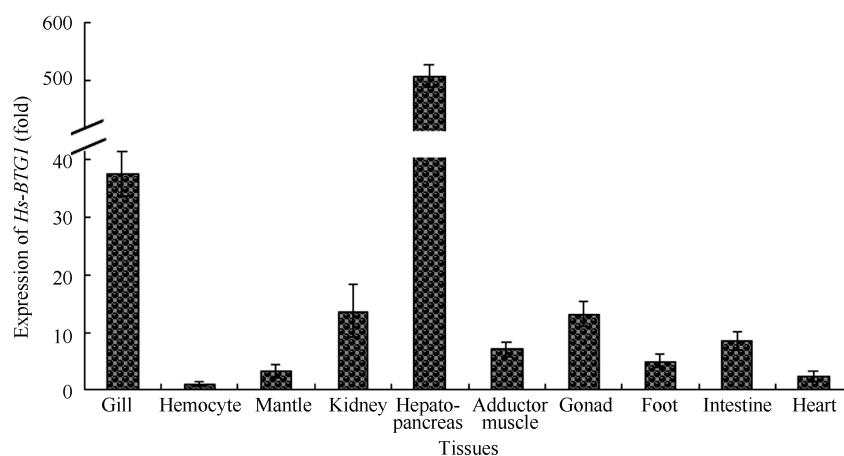


Figure 4 Expression level of *Hs-BTG1* mRNA in different tissues

The vertebrate BTG family was previously reported to be involved in a variety of biological processes, e.g., cell proliferation and differentiation. For example, *BTG1* expression is maximal in the G0/G1 phases of cell cycle and down-regulated throughout the G1 phase (Rouault et al, 1992; Matsuda et al, 2001), suggesting that the expression of *BTG1* may inhibit cell proliferation by

keeping cell cycle arrest in the G0 phase. *In vitro* experiments further demonstrated that overexpression of *BTG1* can inhibit cell proliferation and promote apoptosis (Rodier et al, 2001; Hata et al, 2007; Lee et al, 2003; Matsuda et al, 1992; Rouault et al, 1992). Several studies have shown that *BTG1* is also involved in cell differentiation and early embryonic development, for

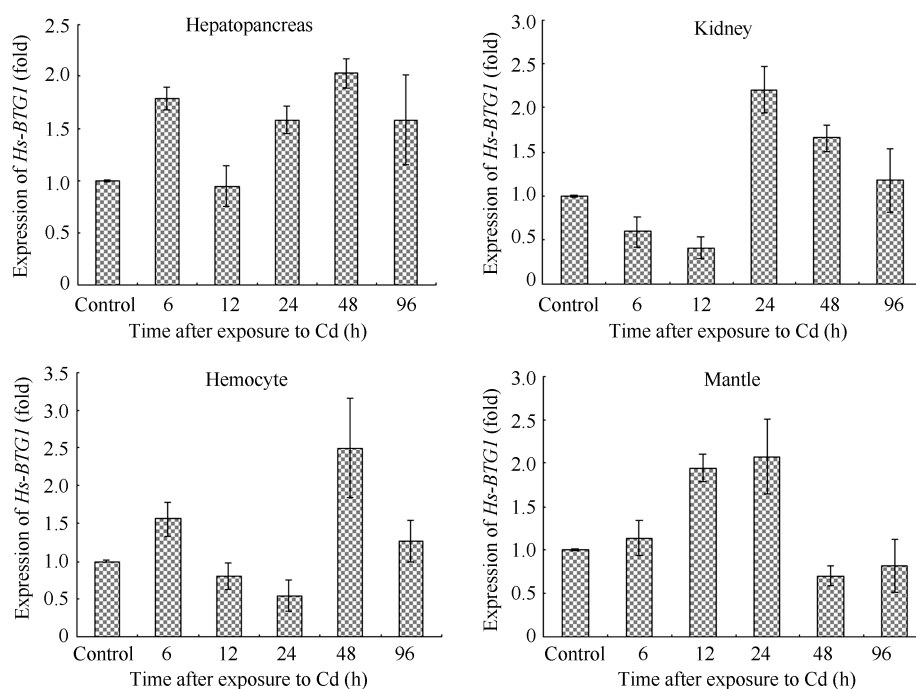


Figure 5 Fold inductions of *Hs-BTG1* mRNA levels in hepatopancreas (a), kidney (b), haemocytes (c) and mantle (d) after exposure to waterborne Cd exposure (5 mg/L)

Data represent group mean fold induction \pm SD ($n=3$ in each time). Asterisks indicate mean fold induction, which are significantly ($P < 0.05$) different from control values (0.005 mg/L).

example in regulating angiogenesis (Iwai *et al.*, 2004), promoting muscle cell differentiation (Busson *et al.*, 2005), and the gastrulation movement in early *Xenopus* embryo (Wessely *et al.*, 2005).

Several lines of evidence suggest that the biological function of BTG is correlated to its expression, such as in adult pigs and sheep where constitutive expression of BTG1 could be detected in several different tissues (Feng *et al.*, 2011; Zhang *et al.*, 2009). Another study found that the highest expression of human *BTG2* was in the renal proximal tubule, alveolar epithelial cells and prostate basal cell layer (Melamed *et al.*, 2002). During the early embryonic development of *Xenopus*, abnormal *XBTG1* expression may cause embryonic gastrulation failure (Saka *et al.*, 2000), while overexpression of a homologous gene *x-BTG-x* may induce a double axis of the embryo (Wessely *et al.*, 2005). Moreover, expression of *BTG-b* was detectable in embryonic induction region, as well as the forebrain, hindbrain and paraxial mesoderm in zebrafish (Sakaguchi *et al.*, 2001). Meanwhile, the highest level of *BTG1* expression in adult grass carp was found in the liver (Fu *et al.*, 2012), consistent with our present results, suggesting that a maintained high expression of *Hs-BTG1* may play an important role for

implementation of normal physiological function of the hepatopancreas.

As we noted earlier, gene expression analysis under various environmental stress factors is a common method of studying candidate gene's corresponding function(s) (Duan *et al.*, 2013; Wang *et al.*, 2011). In the present study, the highest *Hs-BTG1* transcription level was in the hepatopancreas, with the expression being significantly up-regulated when placed under conditions of heavy metal cadmium stress, and expression in other tissues (e.g., blood cells, mantle, and kidney) also increased under stress, though to a lesser extent. Since BTG1 was previously reported to have anti-proliferation and apoptosis effect in higher vertebrates (Corjay *et al.*, 1998; Lee *et al.*, 2003; Rouault *et al.*, 1992), we speculate that *Hs-BTG1* may also be involved in a similar function under cadmium stress. One intriguing observation garnered from this study was that *Hs-BTG1* in kidney tissues was notably up-regulated, but then fell back. A potential explanation may be that the kidney serves a temporary storage of accumulated environmental cadmium. If so, then inhibition of *Hs-BTG1* expression may contribute to proliferation of the renal cells, and then the increased cells would be able to accumulate

greater amounts of cadmium, while conversely a rapid increase of Hs-BTG1 caused by an exceeding threshold level for renal cells tolerance against cadmium may promote cell apoptosis. Though an intriguing possibility,

the exact mechanisms underlying cadmium stress regulation of *Hs-BTG1* in *H. schlegelii*, as well as other freshwater shellfish and invertebrates, remained to be further studied.

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